Modulation of Nrf2-regulated cytoprotective enzymes by crude drugs: role of inducers and inhibitors of Nrf2 in chemoprevention and anticancer therapy

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Abstract

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a potent transcriptional activator and plays a central role in inducible expression of many cytoprotective genes, which produce proteins responsible for the detoxification of chemical carcinogens and the suppression of reactive oxygen species. Under basal conditions, Nrf2 is constantly degraded via the ubiquitin-proteasome pathway in a Kelch like ECH-associated protein 1 (Keap1)-dependent manner. Upon exposure to electrophilic and oxidative stresses, Nrf2 is able to escape Keap1-mediated repression, translocate into the nucleus, and activate the expression of its target genes. It is widely accepted that the induction of Nrf2-regulated enzymes results in protection against massive oxidative stress and chemical carcinogenesis. Nrf2 activators are developed as putative chemopreventive agents. In many human cancers, missense mutations in KEAPI and NRF2 genes have been identified. These mutations are reported to result in permanent Nrf2 activation, leading to overexpression of cytoprotective enzymes. Constitutive induction of Nrf2-regulated enzymes can confer multiple advantages on cancer cells for their proliferation and resistance to chemotherapy. Suppression of abnormal Nrf2 activation is needed, therefore, for a new therapeutic approach against tumors resistant to cytotoxic action of anticancer drugs. However, there is little research to identify Nrf2 inducers and inhibitors from crude drugs. In this article, we investigated whether Nrf2 activity in mammalian cells was enhanced or suppressed by treatment with crude drugs and how modulation of Nrf2-regulated enzymes by crude drugs affected cytotoxic action of chemical compounds.

Key words Crude drug, cytoprotection, cytotoxicity, NAD(P)H:quinone oxidoreductase 1, Nrf2, anticancer drug.

Abbreviations ARE, antioxidant response element; CDNB, 1-chloro-2,4-dinitrobenzene; GR, glutathione reductase; GST, glutathione S-transferase; Keap1, Kelch like ECH-associated protein 1; Nrf2, nuclear factor erythroid 2-related factor 2; NQO1, NAD(P)H:quinone oxidoreductase 1; UGT, uridine 5'-diphospho-glucuronosyltransferase.

General

1) Role of phase 2 detoxifying enzymes in prevention of carcinogenesis

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Common features of human carcinogenesis amenable to preventive interventions include mutagenesis, oxidative stress, and inflammation. Mutagenesis, an initiator of carcinogenesis, reflects DNA damage mediated by electrophilic insults derived from exogenous carcinogens as well as accumulation of reactive oxygen species (ROS). Chronic inflammation is associated with
carcinogenesis because elevated levels of ROS, reactive nitrogen species, and cytokines can lead to DNA damage and cell invasiveness.\textsuperscript{2} Based on these concepts, inhibition of these processes by inducing the expression of electrophile detoxifying enzymes and antioxidant proteins has been postulated to be an effective way to prevent carcinogenesis.\textsuperscript{3}

Many carcinogens undergo metabolic activation to generate reactive intermediates that can directly damage DNA by forming adducts. Phase 1 drug metabolizing enzymes often mediate the metabolic activation of carcinogens, while phase 2 enzymes can compete with the activating cytochrome P450 enzymes by eliminating reactive electrophiles via conjugation with endogenous ligands such as glutathione (GSH) and glucuronic acid, rendering them more water soluble and more readily excretable from the cell and ultimately the body.\textsuperscript{4} Glutathione S-transferases (GSTs), uridine 5' -diphospho-glucuronosyltransferases (UGTs), and NAD (P) H: quinone oxidoreductases (NQOs) are some samples of phase 2 enzymes. The importance of these enzymes in protection against carcinogenesis has been highlighted in several studies with mice lacking specific enzymes and in analyses of human genetic polymorphisms. For example, \textit{GSTP1}-null mice increased lung cancer numbers following exposure to tobacco-related carcinogen benzo [\(\alpha\)]pyrene (B[\(\alpha\])P).\textsuperscript{5} Several human studies confirmed the importance of GST enzymes in carcinogenesis. Analysis of GST polymorphisms and human cancers concluded that \textit{GSTMI}/\textit{GSTTT1} null genotypes and \textit{GSTP1} mutations are associated with increased risk of lung,\textsuperscript{6} prostate,\textsuperscript{7} and colorectal cancers\textsuperscript{8} as well as acute lymphoblastic leukemia.\textsuperscript{9}

NQO1 is a multifunctional antioxidant enzyme. NQO1 plays important roles in protection against quinone-derived reactive intermediates and stabilization of a tumor suppressor p53 against proteasomal degradation.\textsuperscript{10} Targeted disruption of the \textit{NQO1} gene increased susceptibility to B[\(\alpha\])P-induced skin tumorigenesis and benzene-induced toxicity.\textsuperscript{11,12} Furthermore, an investigation of a benzene-exposed population found that individuals with an \textit{NQO1} mutation, which has a negligible NQO1 activity, showed a 7-fold greater risk of bone marrow toxicity.\textsuperscript{13} These experimental and epidemiological studies indicate that phase 2 enzymes play a major role in susceptibility to carcinogenesis in both animals and humans.

2) Nuclear factor erythroid 2-related factor 2 (Nrf2) function and regulation in normal cells

The comprehensive system that detoxifies a range of environmental and endogenous toxins, mutagens, and potential carcinogens is composed of multiple antioxidant and detoxifying enzymes and drug transporters.\textsuperscript{14-16} Nrf2 is a master transcriptional regulator of phase 2 detoxifying enzymes including GSTs, UGTs, and NQOs as well as antioxidant enzymes, such as glutathione reductase (GR) and \(\gamma\)-glutamylcysteine synthetase, which are involved in the generation of reduced glutathione. Additionally, there is a broad spectrum of genes regulated by Nrf2, including molecular chaperones (e.g., heat shock proteins), general cytoprotective mediators (e.g., heme oxygenase-1 (HO-1)), and growth factors (e.g. heparin-binding epidermal growth factor-like growth factor).\textsuperscript{17,18} The expression of cytoprotective enzymes is up-regulated by Nrf2 binding to the antioxidant response element (ARE), a \textit{cis}-acting sequence located in the 5' -flanking region of these genes.\textsuperscript{19}

Under basal redox conditions, Nrf2 is anchored in the cytoplasm through binding to Kelch like ECH-associated protein 1 (Keap1). Keap1 serves as an adaptor protein between Nrf2 and the Culbin3-based E3 ligase ubiquitlation complex, leading to ubiquityllation of Nrf2 and subsequent degradation by the 26S proteasome.\textsuperscript{20,21} Keap1 constitutively suppresses Nrf2 activity in the absence of stress; however, oxidizing conditions and treatment with cancer preventive agents hamper the Keap1-mediated proteasomal degradation of Nrf2, which results in increased nuclear accumulation and subsequent transcriptional induction of target genes that ensure cytoprotection. Indeed, \textit{NRF2}-null mice, which fail to induce many cytoprotective genes, are more susceptible to several diseases in addition to cancer.\textsuperscript{22,23}

Cancer prevention can be achieved through induction of Nrf2-regulated enzymes by using of naturally occurring or synthetic compounds. For example, sulforaphane, which was first isolated from broccoli by monitoring bioassay-guided induction of \textit{NQO1} in murine hepatoma cells,\textsuperscript{24} has been reported to suppress carcinogen-induced tumorigenesis in rodent organs.\textsuperscript{25,26} In human, consumption of broccoli and cruciferous vegetables has
been correlated with the reduction of cancer risk in prostate, colon, and lung.\textsuperscript{27,29} These studies indicate that Nrf2 is a molecular target to exert cancer preventive effects.

3) Constitutive activation of Nrf2 in tumor cells

According to recent studies, evidence is accumulating for frequent somatic mutations of KEAP1 and NRF2 in human cancers. Missense mutations, insertions, and deletions were identified in KEAP1 from the tumors of ten of 54 non-small cell lung cancer patients and in six of 12 cell lines.\textsuperscript{30} Further KEAP1 mutations were identified in additional lung cancer patients; one of eight with small cell lung carcinoma, three of 29 with adenocarcinoma, and one of two with large cell carcinoma were affected.\textsuperscript{31} Although these mutant Keap1 proteins have not all been tested individually, it is believed that they result in inactivation of Keap1 function, leading to constitutive stabilization of Nrf2 and increased expression of cytoprotective enzymes. Mutations in NRF2 have been also found in human cancers. NRF2 point mutations were identified in 11 of 103 patients with lung cancer and in three of 12 patients with head and neck cancers.\textsuperscript{32} The NRF2 mutations clustered around codons for amino acids 24-34 and 75-82, which are critical sites for binding of Keap1. These NRF2 mutations should make Nrf2 constitutively active.

Patients with lung tumors that contain mutant KEAP1 or NRF2 have a worse prognosis than patients with lung tumors lacking such mutations.\textsuperscript{32} KEAP1 or NRF2-mutant lung cancer cells show that constitutive Nrf2 activation and subsequent induction of ARE-driven genes confer resistance against cytotoxic action of anticancer drugs.\textsuperscript{30,31,33} Therefore, it is desirable to devise strategies for antagonizing activated Nrf2 and suppressing overexpression of cytoprotective enzymes to sensitize cancer cells to chemotherapy.

**Nrf2 inducers found in crude drugs**

We examined effects of methanol soluble fractions of crude drugs on Nrf2-regulated enzymes in Clone 9 cells, a cultured cell line from normal rat liver (Fig. 1). After treatment of Clone 9 cells with crude drug extracts for 24 h, cellular NQO1 activity, which is used as indicator of Nrf2 activity, was determined. Among these extracts, NQO1 activity was increased more than 1.5-fold by treatment with Kyokatsu, Koboku, Shini, and Mokko extracts. Of the four extracts, the Kyokatsu extract showed the highest activity, and the activity was the same as for sulforaphane, a well-known potent NQO1 inducer and a cancer chemopreventive agent.

We checked mRNA and protein expression levels of Nrf2-regulated enzymes in Clone 9 cells after treatment with the Kyokatsu extract for several different times (Fig. 2). Real-time PCR and Western blot analyses showed that the expression of GSTs, NQO1, and HO-1 was induced at a transcriptional level by the
Kyokatsu extract. The maximum induction of GSTs and NQO1 was observed at 24 h, while HO-1, which is known as a stress response protein, was induced at an earlier time. To investigate the effect of the Kyokatsu extract on phase 2 and antioxidant enzyme activities, Clone 9 cells were incubated with different concentrations of the extract for 24 h, and then those enzyme activities were measured. As shown in Fig. 3, significant increases in GST, NQO1, UGT, and catalase activities and intracellular GSH contents were confirmed.

Many lines of evidence indicate that the expression of phase 2 enzyme such as GSTs and NQOs is regulated mainly by ARE and that the most effective transcription factor that binds to ARE is Nrf2. To determine whether the Nrf2/ARE pathway actually contributes to the induction of phase 2 and antioxidant enzymes by the Kyokatsu extract, we analyzed nuclear localization of Nrf2 by Western blotting and measured ARE-mediated transcriptional activity based on luciferase reporter assay in Clone 9 cells after treatment with the extract (Fig. 4). When cells were incubated with several different concentrations of the Kyokatsu extract for 6 h, Nrf2 accumulated in the nucleus in a dose-dependent manner. Transient transfection assay using luciferase system showed that the Kyokatsu extract significantly enhanced ARE-luciferase activity. These results indicate that the Kyokatsu extract activated the Nrf2/ARE pathway.

Nrf2-regulated cytoprotective enzymes are involved in the suppression of oxidative stress and the detoxification of electrophilic chemicals. To demonstrate...
the cytoprotective effect of induction of phase 2 detoxifying enzymes via activation of the Nrf2/ARE pathway by the Kyokatsu extract against toxic stress, we verified whether the extract could protect against cell death induced by menadione, 1-chloro-2,4-dinitrobenzene (CDNB), and ethacrynic acid, which are cytotoxic compounds known as generators of oxidative and electrophilic stress (Fig. 5). When cells pretreated with vehicle (methanol) were exposed to those cytotoxic compounds for 24 h, cell viabilities were markedly decreased to less than 10% of control. In contrast, the massive cytotoxicity was significantly suppressed in cells pretreated with the Kyokatsu extract. In accordance with these results, quantitative analysis by HPLC revealed that the amounts of the detoxified metabolites including glucuronides and GSH conjugates of those cytotoxic compounds were greater in cells pretreated with the Kyokatsu extract compared with those in control cells (data not shown). These data indicate that induction of Nrf2-regulated enzymes by the Kyokatsu extract can lead to protection against chemical-induced cytotoxicity through the enhancement of its detoxification pathway.

![Fig. 4](image_url) Activation of the Nrf2/ARE pathway by the Kyokatsu extract. (A), Western blot analysis of nuclear Nrf2 accumulated by treatment with the Kyokatsu extract in Clone 9 cells. (B), Enhancement of ARE-luciferase activity in Kyokatsu extract-treated Clone 9 cells. Values are represented as mean ± SD (n = 3). *Significantly different from control at p < 0.05.

![Fig. 5](image_url) Protection of Clone 9 cells by pretreatment with the Kyokatsu extract against cell death induced by cytotoxic compounds. Cells were pretreated with the Kyokatsu extract (100 µg/ml) for 24 h and then were exposed to cytotoxic compounds. Data are expressed as mean ± SD of three different experiments. *Significantly different from each vehicle control at p < 0.05.

**Nrf2 inhibitors found in crude drugs**

Recent studies have demonstrated that certain cancer cell lines that overexpress Nrf2-regulated enzymes because of KEAPI or NRF2 gene mutations acquire multiple advantages for proliferation and resistance to chemotherapy. In the present article, we attempted to select crude drugs that can inhibit constitutive activation of Nrf2 in human lung cancer A549 cells, which have a point mutation in the KEAPI gene causing amino acid substitution of G333C. Methanol soluble fractions of crude drugs were screened for Nrf2 inhibitors that have the ability to suppress NQO1 activity in A549 cells (Fig. 6). Of all the extracts examined, NQO1 activity fell to less than 50% of its original value after treatment with Mao or Keihi extract. The Keihi extract was found to be the most potent inhibitor of NQO1.

To examine how treatment with the Keihi extract affects Nrf2-regulated enzymes, we assessed the level of phase 2 and antioxidant enzyme activity in A549 cells treated with the extract. As shown in Fig. 7, both these parameters were significantly decreased after treatment.
with the Keihi extract, including a large reduction in the level of intracellular GSH. Western blot analysis showed that treatment with the Keihi extract reduced the expression of cytosolic GSTP1 and NQO1 and nuclear Nrf2 (Fig. 8). These results suggested that the decrease in GST and NQO1 activities was caused by Keihi-mediated down-regulation of enzyme expression, not by Keihi-mediated direct inhibition of enzyme activity.

We reasoned that the Keihi extract effectively inhibits Nrf2 expression and Nrf2-regulated enzyme activity in A549 cells. If so, this would suggest an application for the Keihi extract in sensitizing lung cancer cells to anticancer drugs. Therefore, we tested the hypothesis by treating cells with doxorubicin, cisplatin, and etoposide in the presence or absence of the Keihi extract (Fig. 9). Our data showed that the cytotoxicities of anticancer drugs were significantly enhanced in the presence of the Keihi extract.

**Fig. 6** Effect of crude drug extracts on NQO1 activity in A549 cells. Cells were treated for 24 h with 100 μg/ml of methanol soluble fraction of each crude drug. Data represent the mean of duplicate determinations.

**Fig. 7** Decreases in Nrf2-regulated enzyme activity in A549 cells treated with the Keihi extract. Cells were treated with the indicated concentrations of the Keihi extract for 24 h. Data are expressed as mean ± SD of three different experiments. *Significantly different from control at p < 0.05.

**Fig. 8** Suppression of Nrf2 protein expression in A549 cells by treatment with the Keihi extract. Western blot analysis of cytosolic and nuclear fractions in A549 cells treated with the indicated concentrations of the Keihi extract for 24 h.
Conclusion and perspectives

Role of the Keap1-Nrf2 pathway in cancer prevention and chemoresistance has been well established in various recent studies. According to those research, Nrf2 is a molecular target of chemopreventive and chemosensitive agents. However, to the best of our knowledge, there has been no research to perform screening procedures for finding Nrf2 inducers and inhibitors concurrently and demonstrate the pharmaceutical usefulness of Nrf2 modulators from herbs. Based on the results of our screening test, we found that Kyokatsu or Keihi extracts might act as an Nrf2 inducer or inhibitor and exert the chemopreventive or chemosensitive effect, respectively. It would be interesting to investigate whether Kampo products containing Kyokatsu or Keihi can affect the Keap1-Nrf2 pathway and modulate the expression of ARE-driven cytoprotective genes.

The notion that constitutively active Nrf2 might aid tumorigenesis recalls an older body of literature in which high dose of phenolic antioxidants, such as butylated hydroxyanisole, which has the ability to activate Nrf2, were reported to promote tumor development of the forestomach in rodents. The fact that somatic mutations occur in tumors causing constitutive Nrf2 activation, coupled with the older data about high-dose treatment of animals with phenolic antioxidants, should sound a cautionary note. Although there is no evidence to suggest that Nrf2 inducers have adverse impacts on tumorigenesis in humans, treatment for long periods with doses of Nrf2 inducers that continuously cause maximal Nrf2 activation should be avoided in humans.

Chemoresistance by unrestrained Nrf2 activation in cancer cells is likely to be associated with poor prognosis and lower postoperative disease-free survival rates in patients. Discovery and development of Nrf2 inhibitors should make a critical contribution to improved chemotherapy. A recent study reported that disruption of Nrf2 by a specific siRNA inhibits tumor growth and increases the efficacy of chemotherapy. However, there are only a few reports that investigated whether small molecule compounds could suppress constitutive activation of Nrf2. Our in vitro data demonstrated that lung cancer cells acquiring resistance to anticancer drugs due to Nrf2 overexpression became susceptibility to killing by the combined action of an anticancer drug with the

Fig. 9 Increased sensitivity of A549 cells to cytotoxic action of anticancer drugs by treatment with the Keihi extract. Cells were incubated with anticancer drugs in the presence of 300 μg/ml of the Keihi extract for 24 h. Data represent mean ± SD of three different experiments. *Significantly different from control cells at each concentration at p < 0.05.
Keihi extract. Discovery and development of Nrf2 inhibitors should make a critical contribution to improved cancer therapy. Identification of small molecule Nrf2 inhibitors contained in the Keihi extract is our ongoing project.

Acknowledgements

We thank Tsumura Co. Ltd. for providing the crude drugs. We thank Ms. Ayano Itoi, Ms. Ayako Kawana, Ms. Mai Shimoda and Mr. Shin Saito (Tokyo University of Pharmacy and Life Sciences) for their help with our experiments.

References


34) Ito, N., Fukushima, S. and Tsuda, H.: Carcinogenicity